Prevalence of cervical cancer among Pakistani women and its proteome's dynamic alteration

Abstract

Cervical cancer is the second cause of death in malignancies among women all around the world. Previous studies reveal that HPV is main cause of this cancer. Survival is low because of late diagnosis.

Method: In our studies history of 85 patient were collected according their age, marital status, class. The HPV was detected by using general primer GP5/GP5 in PCR. The type HPV-16 and HPV 18 were detected. Proteomic analysis of cervical cancer was performed by SDS-PAGE electrolysis and 2-Dimensional Gel Electrophoresis. The quantitative analysis was done by Image Master Melanie Platinum Software 7.0.

Result: It was inferred that cervical cancer is mostly found in poor class, married and old age women. The risk factor seen was less education, lack of awareness, low socio-economic status and late diagnosis. Protein expression in a disease can open a sight in understanding cause of cancer. It can helpful in diagnosis and treatment of cervical cancer. When protein expression was evaluated in cervical cancer there was a significant difference present in normal and cancerous protein expression.

Conclusion: The plasma protein which were found to be up-regulated were C3, MMP-9 and Cytokeratin 19. In addition, the protein expression of cervical cancer tissue showed upregulation of SCCA, HSP27 and 14-3-3 protein. So, these proteins can be helpful biomarkers but further investigation is required for confirmation of validity of these protein biomarkers.

Key words: Cervical Cancer, HPV, SDS, Two-dimensional Electrophoresis

Introduction

Cervical cancer arises from the cervix, its main cause is uncontrolled growth of cell which can spread to the various portion around the cervix and elsewhere in the body. In malignancies cervical cancer is at second number of death cause among women. In can be interpreted from the work of Jamel et al 2011 that incidence of cervical cancer is highest in Africa that is about 26-34 per 100, 000. In addition, it is descending in south-central Asia, South America, Melanesia and Caribbean from 24.6 to 20. On the other hand, it lies between 6.0 to 15 south, eastern Asia and Europe. However, it is least found in northern America, New Zealand, Australia and Western Asia that is 4 to 5.7 as indicated in figure 1.

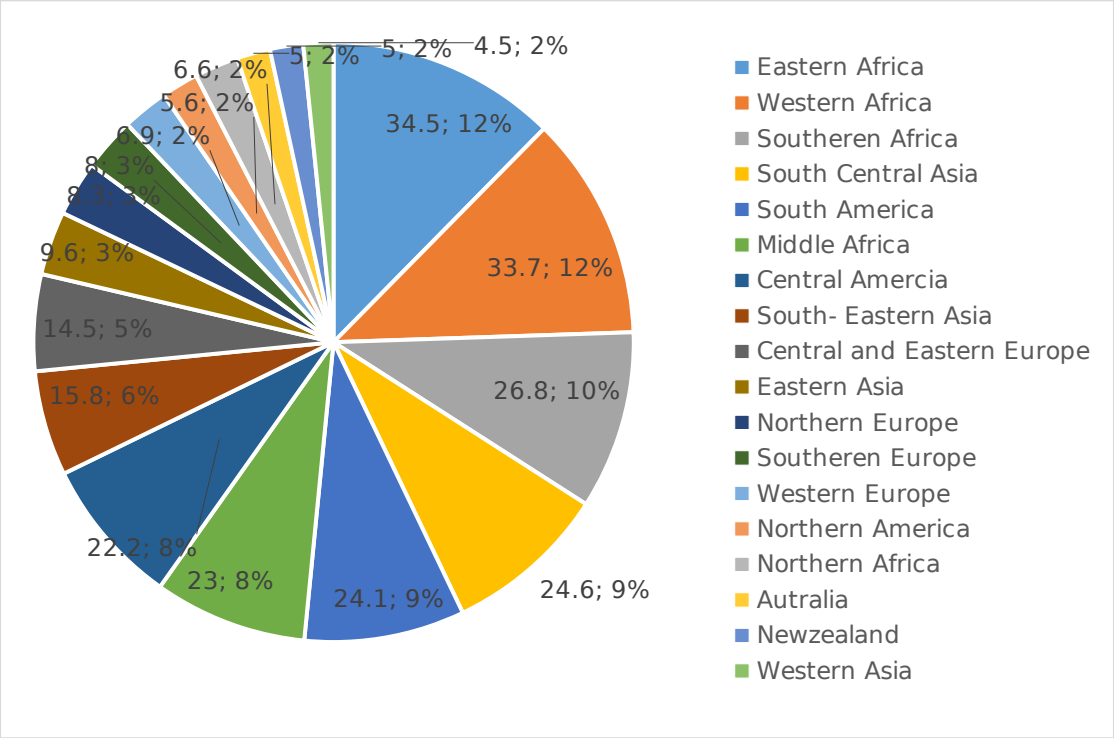

Figure 1. worldwide prevalance of Cervical Cancer

One of the most conjoint sexually transmitted viruses among men and women in Western countries is the humanpapillomavirus (Schoell et al 1991). It is studies that there is strong link between HPV infection and cervical lesion leading to cancer (Jenicek et al 2001). There are over 100 different subtypes of HPV, which are distinguished by variations in their genetic sequence. On the basis of their oncogenic potential, HPV subtypes are classified into high- and low-risk (Bosch et al 1995). Infection with high-risk HPV genotypes leads to an increased risk of cervical carcinoma. Among the HPV subtypes that infect the anogenital tract, subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, and 69 have been defined as high-risk for

cervical carcinoma (Hausen et al 1996). The other types are generally not causing severe form of cancer (Cuzick et al 1995).
All HPVs have an 8-kb circular genome enclosed in a capsid shell comprising the major and minor capsid proteins L1 and L2, respectively. Purified L1 protein will self-assemble to form empty shells that resemble a virus, called virus-like particles (VLPs). In addition to the structural genes (L1 and L2), the genome encodes several early genes (E1, E2, E4, E5, E6, and E7) that enable viral transcription and replication and interact with the host genome. Immortalization and transformation functions are associated with the E6 and E7 genes.HPV genome comprise of up-stream regulatory region or long control regulatory region, early gene region and late gene region. Early gene region consists of E1, E2, E6 and E7. Late region contains L1 and L2 represented by figure 2.

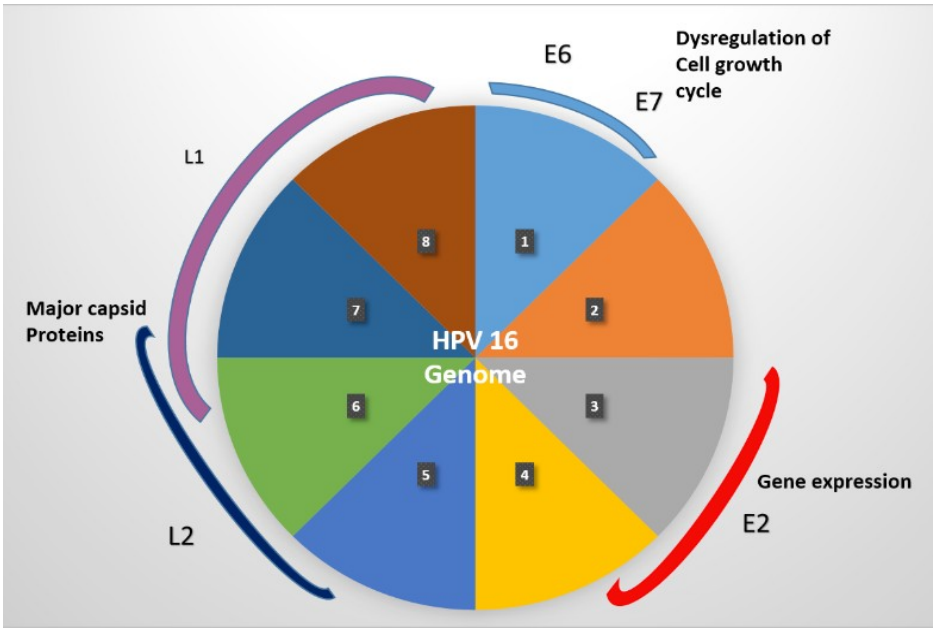

Figure 2. Genome of HPV16

As these protein are involved in cell cycle regulation, signal transduction, DNA replication, and cellular proliferation (Wentzensen et al 2007). When oncogene of HPV E6 and E7 bind with the host regulatory protein than it results into degradation of p53 tumor suppressor gene. Consequently, the retinoblastoma protein gets inactivated. It leads to the dysregulation of cell cycle narrated in figure 3. (Charllote et al 2011) (Dehn et al 2007).

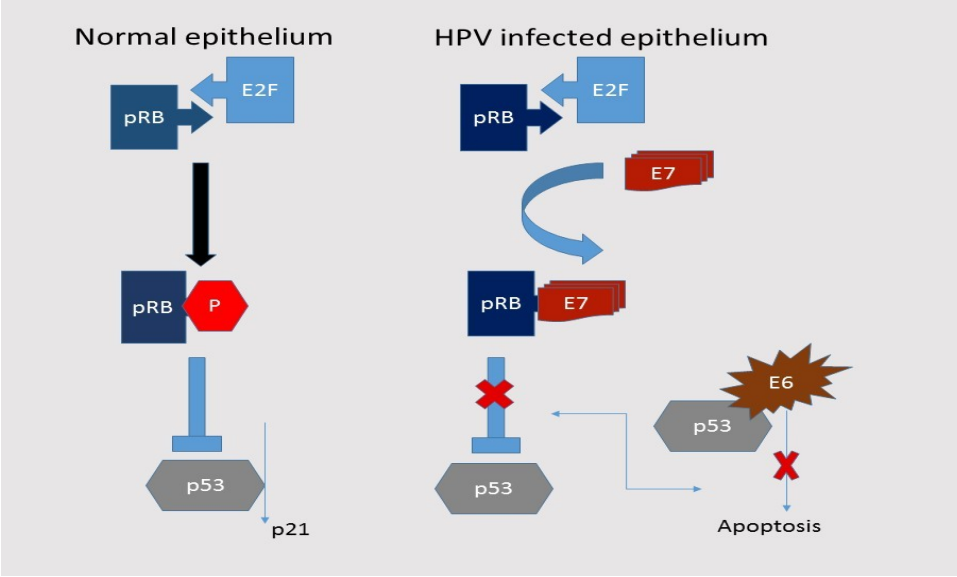

Figure 3. Mode of action of HPV

Proteomic techniques have significant role in the identification of protein evaluation in a cancerous cell. It can be used to target the protein which are involved in the molecular events in the progression of cancer. Techniques used in proteomics are two-dimensional electrophoresis and matrix-assisted laser desorption and ionization time of flight mass spectrometry (MALDI-TOF-MS). Candidate proteins were also identified by the use of RT-PCR, Western blotting, and immunohistochemical staining. These proteins may provide worthwhile information corresponding to early detection of cervical SCC. Plasma, serum and tissue proteins which were considered candidate tumor marker in previous studied are shown in table 1.

Table 1. Previous research proteins

Plasma Proteins	
C3 C4 Cytokeratin 19 Tetranectin Clusterin	2009
Ceruloplasmin Complement C3 Afamin precursor Alpha-1-B glycoprotein Chain A, transferrin Alpha-fibrinogen precursor Chain I, crystal structure of	2008

Antithrombin Apolipoprotein A-IV precursor	
Serum proteins	
C3 A1BG	2014
MMP-2 MMP-9	2009 2009
Tissue proteins	
Glutathione S-transferase heat shock protein 27 (Tropomyosin 3) SCC-1 SCC-2 Annexin A2 Annexin A5	2006
Pigment epithelium derived factor Annexin A2 and A5 Keratin 19 and 20 Heat shock protein 27 Smooth muscle protein 22 alpha, α-enolase Squamous cell carcinoma antigen 1 and 2 Glutathione *S*-transferase, apolipoprotein a1 14-3-3 Proteins	2005
Inflammatory factor 1 (AIF-1) Actine-like protein 2 (ALP2), brain type Fatty acid-binding protein (B-FABP) NCK adaptor protein 1 (NCK-1) Islet cell autoantigen 1 (ICA69) Cationic trypsinogen (PRSS1) cyclin-Dependent kinase 4 (CDK4),	

Material and Method

The research objectives strictly followed national and institutional regulations.

Phenol-Chloroform method

Table 2

Solution	Preparation (volume was made

	up to 50ml of distill water.)
Solution A	5.45 g of 0.32 M Sucrose, 0.5ml of 10 mM Tris, 0.25 ml of 5 ml $MgCl_2$ and 0.5 ml of 1 % Triton X-100
solution B	0.5 ml of 10 mM Tris, 5 ml of 100 mM NaCl and 0.1 ml of 2 mM EDTA
Solution C	10 ml of freshly prepared phenol and 0.1 ml of 10 mM Tris (Volume was not changed)
Solution D	24 ml of chloroform and 1 ml of isoamyl alcohol
DDB	0.5 ml of 10 mM Tris and 0.005 ml of 0.1 mM EDTA

Negative DNA was extracted from normal blood samples. In a 1.5 ml Eppendorf 500ul blood and 500ul solution A was taken. It remains at room temperature for 15 minutes to remove RBCs. This solution was centrifuged for one minute at 13000 rpm. The supernatant was removed and pallet was mixed with 400ul solution A. again centrifugation was performed for one minute. This step is repeated 2, 3 times until RBCs remove completely. After this pallet was mixed with 400ul solution B. This solution was suspended in 12ul SDS 20% solution, 20ul protein kinase and keep it at 50 C incubator for 2 days. After this 500ul of solution C and same amount of solution was added into the sample and centrifuged for 5 minutes at 13000 rpm. Aqueos layer was formed which were transferred into new eppendorf. Again, solution D was added in equal amount and centrifuged for 10 minutes at 13000 rpm. The step is repeated. Resultant aques layer was taken in new eppendorf. 3 M sodium acetate of volume 55 µL having pH 6 and ~ 500 ml of chilled isopropanol were added to the upper layer and the tubes were inverted several times.DNA thread was found. It was centrifuged for 10 minutes. And washed with 70% ethanol.The pallet was air dried and 50ul DDB was added.(table 2)

The viral DNA was amplified by PCR by using GP5/GP6 primer given in table 3. 25ul reaction mixture was prepared in PCR tubes. First of all, PCR 17 ul water was added. Moreover, 2.5 µL Buffer (MgCl, KCl), 0.5 µL sNTPs, 0.5 µL forward primer, 0.5 µL reverse primer, 3 µL $MgCl_2$ and .5 µL DNA samples were added. In the end 0.5 ul taq polymerase was added. PCR condition was set according to table for 30 cycles.

Table *3*. General primer with sequence and product size

Primer	**Sequence**	**Target**	**Product**

			(bp)
PC03 PC04	ACACAACTGTGTTCACTAGC CAACTTCATCCACGTTCACC	β-globin	110
$GP5_{+/}$	+ 5' TTTGTTACTGTGGTAGATAC 3' - 5' GAAAAATAAACTGTAAATCA 3'	L1	150
TS 16	+ 5' TGCTAGTGCTTATGCAGCAA 3' - 5' ATTTACTGCAACATTGGTAC 3'	E6 HPV 16	96
TS 18	+ 5' AAGGATGCTGCACCGGCTGA 3' - 5' CACGCACACGCTTGGCAGGT 3'	L1 HPV 18	115

Table 4. Primer with PCR condition

Primer Name	**Hot Start**	**Denaturation**	**Annealing**	**Extension**	**Final Extension**
PC03/PC04	94 °C, 4 min	94 °C, 30 s	54 °C, 45 s	72 °C, 30 s	7 min, 72 °C
GP5+/ GP6+	94 °C, 4 min	94 °C, 30 s	37°C, 45 s	72 °C, 30 s	7 min, 72 °C
TS 16/18	94 °C, 4 min	94°C, 45 s	55 °C, 45 s	72 °C, 30 s	7 min, 72 °C

Table 4 narrates the PCR temperature of different steps. The PCR products were visualized on 2 % agarose gel. TAE buffer 10 x stocks were prepared by adding the glacial acetic acid 10.9 ml, Tris-base 48.4 gm, 2.92 gm Na_2EDTA and volume was made upto 1 liter with distilled water. pH of the solution was adjusted upto 8.29. In a 100 ml reagent bottle 0.5 gm of agarose was suspended in 50 ml of 1x TAE buffer (0.4 M Tris base, 0.01 M EDTA, and 0.2 M acetic acid, pH 8.29), swirled and heated in a microwave oven at low setting until agarose dissolved and solution became homogeneous. The gel was then cooled to 60°C and 5 µl of ethidium bromide (10mg/ml) was added. The liquified gel was poured on to a tidy, leveled gel tray with comb. After the gel solidification the comb was carefully removed and the gel was placed in a mini gel apparatus. 1x TAE buffer was poured in the tank until the surface of the gel was just submerged.

To load DNA samples 6x gel loading buffer (glycerol 3.0 ml, bromophenol blue 30.0 mg, made volume upto 10 ml with TE buffer) was used. The DNA samples after PCR at different annealing temperatures and the DNA size marker (1kb Fermentas ladder) were loaded in the wells of 1.5% agarose gel and were subjected to electrophoresis at 80V for 90 minutes. To verify the presence and size of the product, the gel was observed under UV transilluminator. Photographs were captured using gel documentation system.

Plasma sample was prepared by taking 5ml of blood in EDTA vials. These were centrifuged at 1300rpm for 10 minutes. The plasma was separated. A protein gel casing system (Mini-PROTEAN 3, BIO-RAD) was employed in the preparation of gels [gel size 8 cm (W) and 7.3 cm (H)] using glass plates (having permanently bonded spacer of 0.5 mm thickness). For the preparation of gel assembly glass plates were placed into the holder and tighten with the help of clamps. After setting the gel system resolving solution was poured between the glass plates, leaving space of 1 cm for stacking gel. SDS-PAGE was carried out according to the method of Laemmli. 12% SDS gel was made. In fixative solution (90% ethanol and 10% glacial acetic acid) gel was incubated overnight. In staining solution (.6g commasie G250 was added in100 ml of distilled water. 50g ammonium persulphate was added in 100ml of distilled water. 50 ml phosphoric acid wad added. The three mixtures were mixed and total volume was made upto 400ml). 40ml staining solution and 10 ml methanol was mixed) gel was incubated overnight. In addition, detaining solution (50ml methanol and 70 ml acetic acid was taken and volume made upto 1000ml) incubated the gel overnight.

Tissue was ground in mortar pestle. Powder was suspended in 10% TCA-Acetone. DTT 10mM Sonication was done on ice for 5 minutes. Incubation was done at -20 C for 45 minutes. Sample were centrifuge for 2000g at 4C for 25 minutes. Pallet was washed with acetone (cold) three times. Pallet was dissolved in lysis buffer (8M urea, 2M thiourea, 40Mm tris, 0.1Mm DTT, 4% CHAPS,1M PMSF, P1). Sonication was done for 5 minutes. Sample was centrifuge at 4000g for 30 minutes. Supernatant was stored at -70C. IPG strips were rehydrated in rehydration buffer 5ml (Urea 2.4mg, Thio urea 760mg, DTT 100mg, CHAPS 50mg, Servalyte 45ul few grains of bromophenol) blue according to manufacturer's instruction. 200ul sample and same amount of rehydration buffer was added. IPG strip was gently placed.2-3 ml of mineral oil was overlaid. Lid were placed for 11 to 16 hours. The condition of isoelectric focusing were as start voltage 0 V end voltage 8000 temperature was 20C. Prior to second dimension the strips were placed in equilibrium buffer PH Buffer 8.8, 6.7ml, Urea 72.07g, Glycerol 60 ml, SDS 4g, Bromophenol blue few drops for 15 to 20 minutes. The running conditions were 200 V for 55 minutes.

Results

Samples were collected from Institute of Nuclear medicine and Oncology Hospital Lahore and Aziz Bhatti Shaheed Hospital Gujrat. History of 83 patients were achieved. In which their past illness, personal, social, family history, sign and symptoms, previous investigation, histopathology and staging were evaluated.
The risk factors which were considered were marital status, social class, no of pregnancies and age as indicated in table 5. The symptoms which were accessed were pain, bleeding and menopause. The result shows that it is highly present in married women. Which can become a reason to conclude that it is sexually transmitted disease. Moreover, it is more present in lower class. It can be said that less awareness and poor hygiene may cause this cancer. When estimate was done on the basis of age it was seen that it is present in the women of age greater than 35. It was also observed that mostly patient have no of pregnancies greater than 4.

Table 5. Percentage and frequency of risk factors

Marital status	**frequency**	**percentage**
married	70	84
widow	11	13
unmarried	2	2
lower class	75	90
upper class	8	10
Symptoms		
pain	13	16
menopause	17	21
pelvic discharge	31	38
bleeding	42	56

When these factors were accessed through statistical formulae through Minitab. The result was as shown in table 6. And table 7.

Table 6. Statistical evaluation of factors

factor	**target**	**mean**	**std**	**p-value**
Age	age>35	50	10.74	>0.001
no of pregnancy	>4	5	2.14	>0.001

Table 7. Representation of p-value of factors and symptoms

factrors	**frequency**	**percentage**	**p-value**	**p-value**
married	70	84	<0.05	>0.001
low class	75	90	<0.05	>0.001

Symptoms				
pain	13	16.25	>0.05	0.999
menopause	17	21.25	>0.05	0.970
pelvic discharge	31	38.75	<0.05	>0.001
bleeding	45	56.25	<0.05	>0.001

These results indicate that significant symptoms are pelvic discharge and bleeding

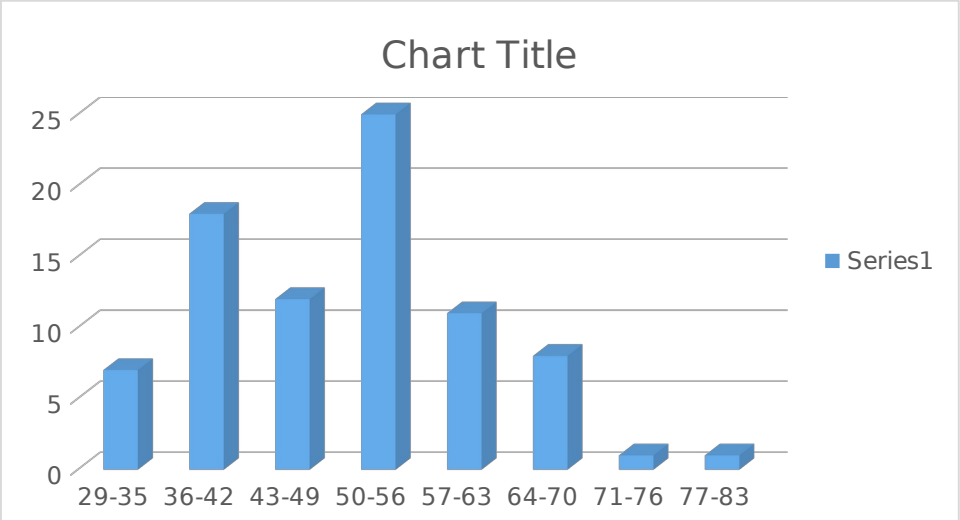

Figure 4.

Figure 4 represents HPV infection with respect to age. The analysis shows that the cancer is higher in the women of age among the 40 to 70. Whereas the occurrence in women age below 30 are very rare.

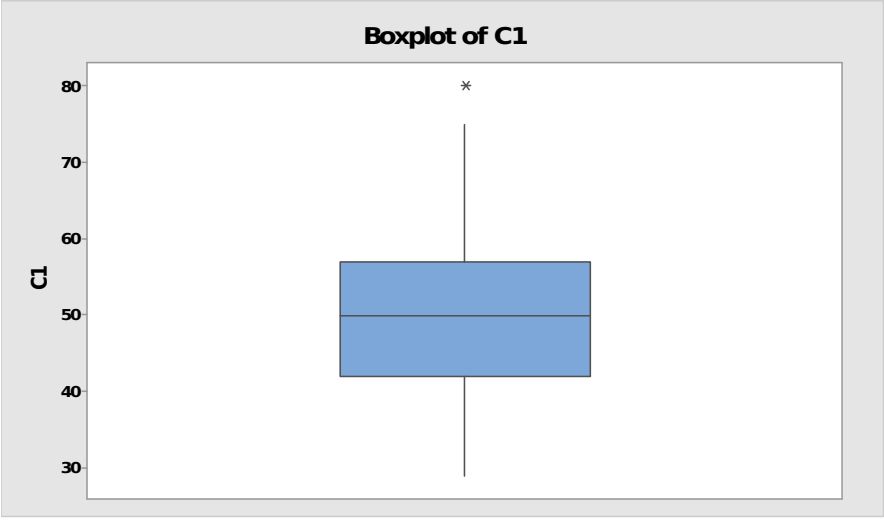

Figure 5.

Figure 5 indicates the Box plot indicate the occurrence of cancer among different aged women

In history the stages of cancer among women were observed.

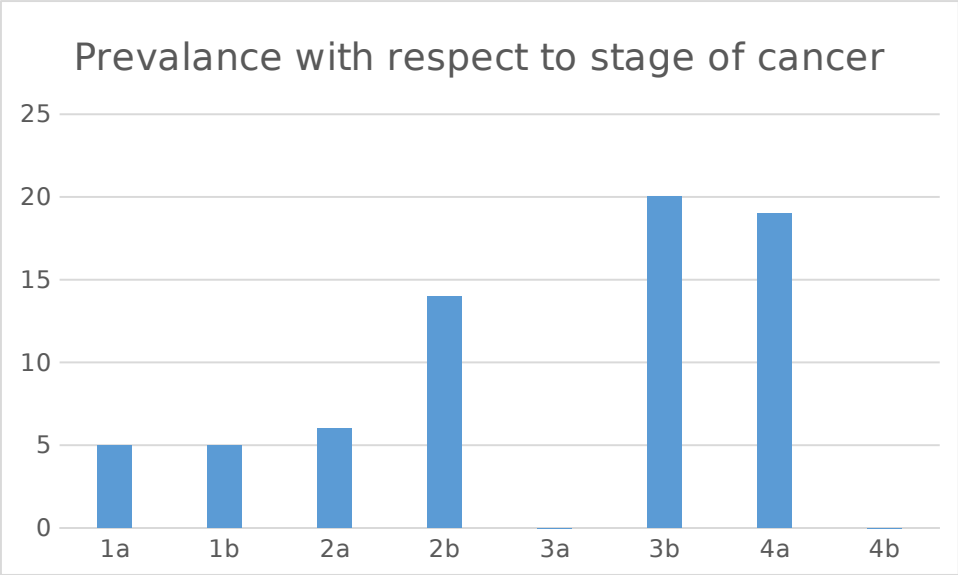

Figure 6.

Histogram indicate the women distribution according to stages of cancer as shown in figure 6.

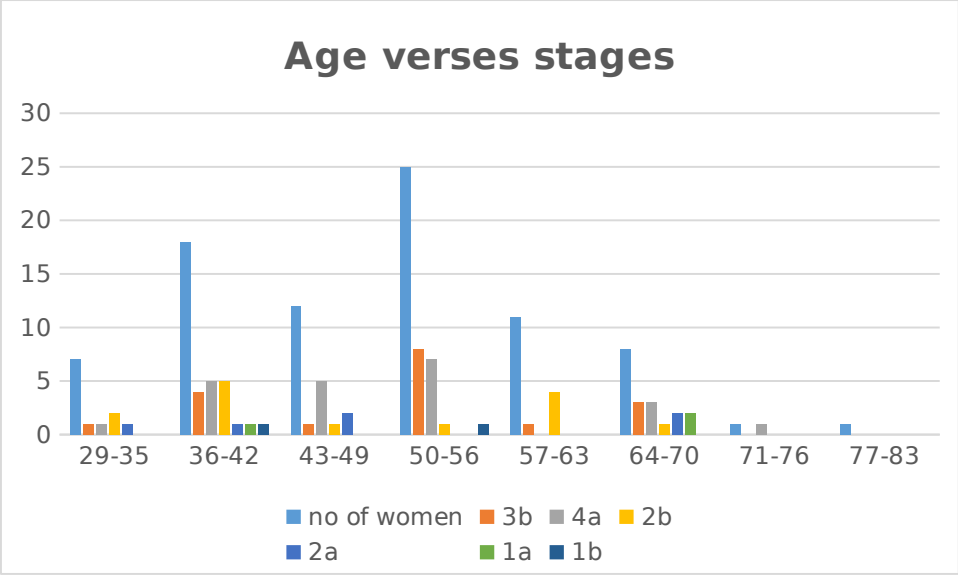

Figure 7.

Figure 7 represent the bar graph represents women ages and their disease staging

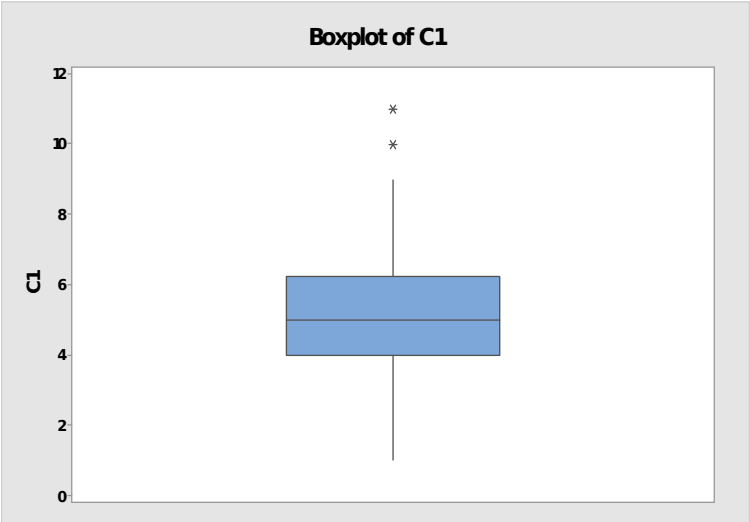

Figure 8.

Figure 8 delineate the box plot indicate the risk factor that is no of pregnancy.

HPV detection in cervical cancer samples

The detection of HPV was performed by PCR using GP5+/6+ primer. Specific condition was applied given in table2. L1 band of 150 base pair was appeared.

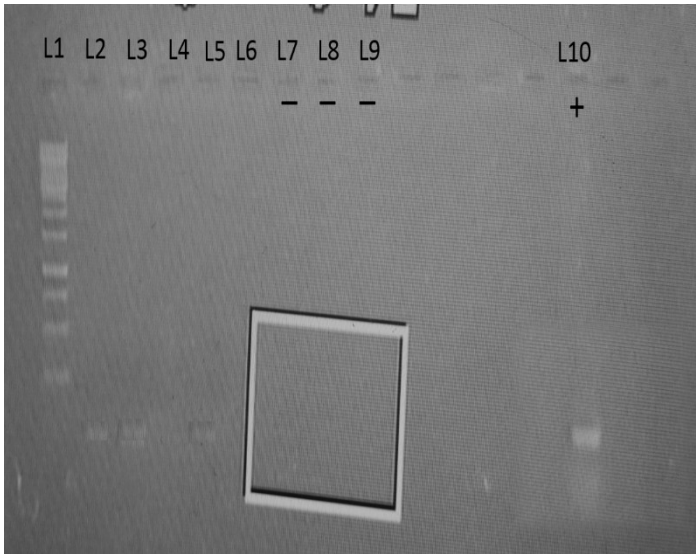

Figure 9.

Figure 9 delineate the HPV detection by PCR. Lane 1 shows the DNA molecular ladder of 1kb. Lane 10 indicates the confirm positive HPV L1

DNA. L2, L3 and L4 shows the presence of positive DNA. On the other hand, L7, L8 and L9 are the negative controls having negative DNA from normal samples.

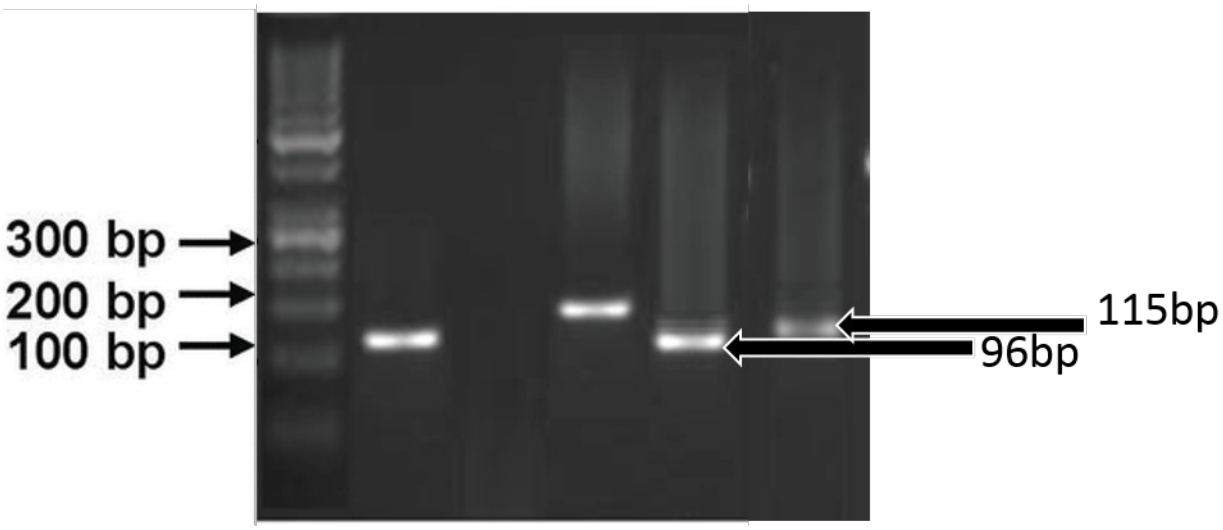

Figure 10.

Figure 10 indicates the detection of HPV by using general primer GP5+/6+. Positive DNA shows band of 150bp. By using TS 16 primer, genotype 16 depicts band of 96 bp and genotype 18 show band of 115bp.

SDS-Gel Electrophoresis

15 microgram plasma protein of one normal and two cancerous samples were loaded along with the protein ladder. The analysis was done in software gene tools from syngene. The difference was observed in the proteins of molecular weight of 200kDa to 10kDa.

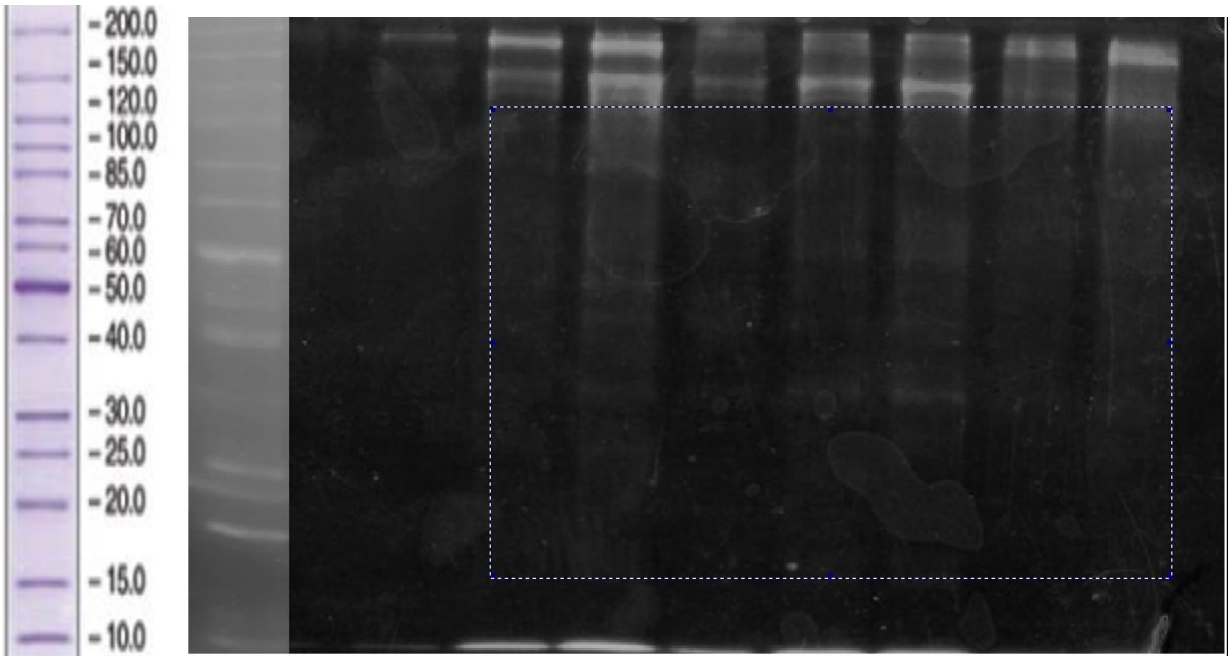

Figure 11.

Figure 11 is representing the normal sample and cancerous samples proteins. Track 4 is 15 micrograms of protein of normal sample whereas track 6 is the same amount of protein of cancerous sample.

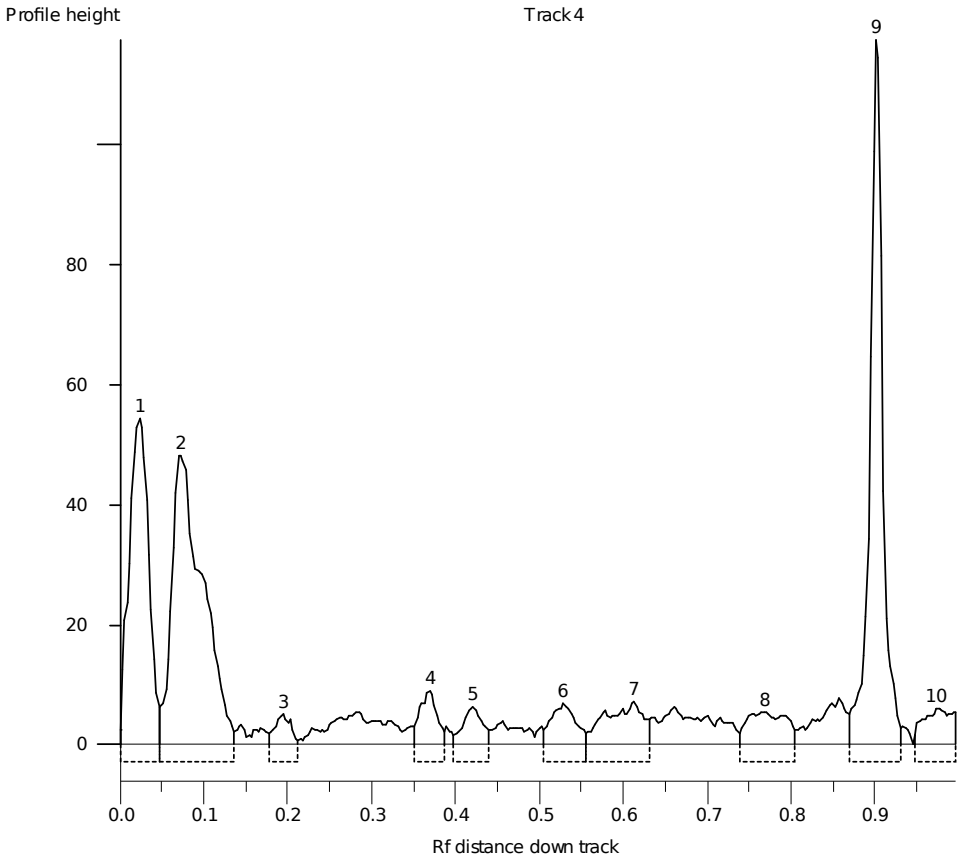

Figure 12

Figure 12 indicates the graphic representation of Rf values of under observation proteins.

Table 8.

Track 4			
Number	**Mol. weight**	**Height**	**Raw vol.**
1	187	54.437	23387.77
2	150	48.271	32785.58
3	78	5.024	1788.69
4	52	9.127	3299.64
5	48	6.391	2617.60
6	31	6.843	3596.33
7	26	7.206	5753.04
8	20	5.594	4759.36

9	10	117.384	32471.49
10	7	6.148	3840.16

Graph 1

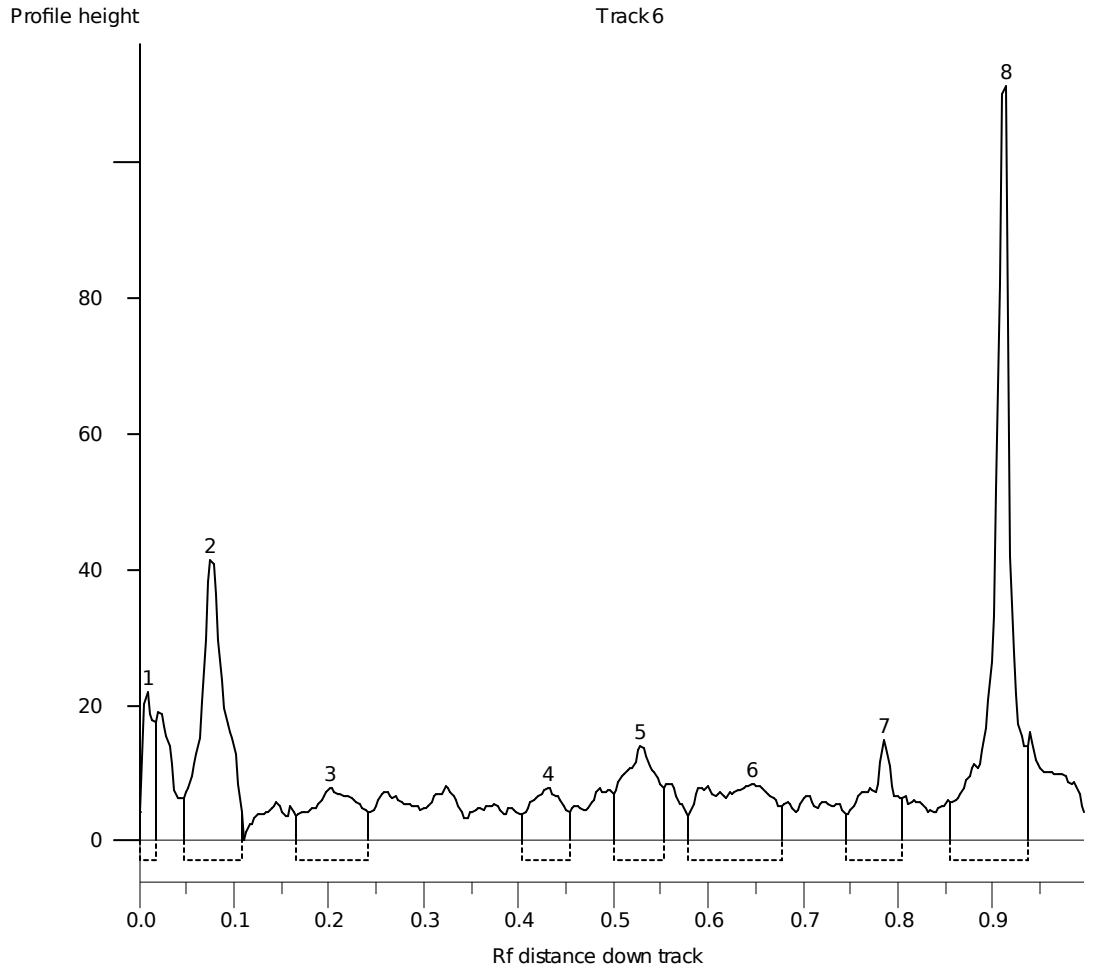

Figure 13

Track 6			
Number	**Mol. weight**	**Height**	**Raw vol.**
1	187	221.864	44676.38
2	150	41.535	19291.00
3	78	7.833	6906.68
4	48	7.695	4798.89
5	32	14.003	8731.78
6	31	8.472	11042.34

7	17	14.847	7470.91
8	10	111.404	36146.23

Figure 13 indicates the graphic representation of Rf values of under observation proteins.

It can be inferred from the Gel Analyzer analyzer analysis in plasma protein the C3 protein 187kDa, MMP-9 78kDa and Cytokeratin 19 31kDa upregulated in cancerous sample (table 8).

4.10. Two-Dimensional Gel Electrophoresis

The gels were analyzed by software Image Master (Melanie 7). The gels were cropped and contrast was adjusted in the software. Spots were detected. There were 491 spots in cancerous sample and 328 in normal sample.

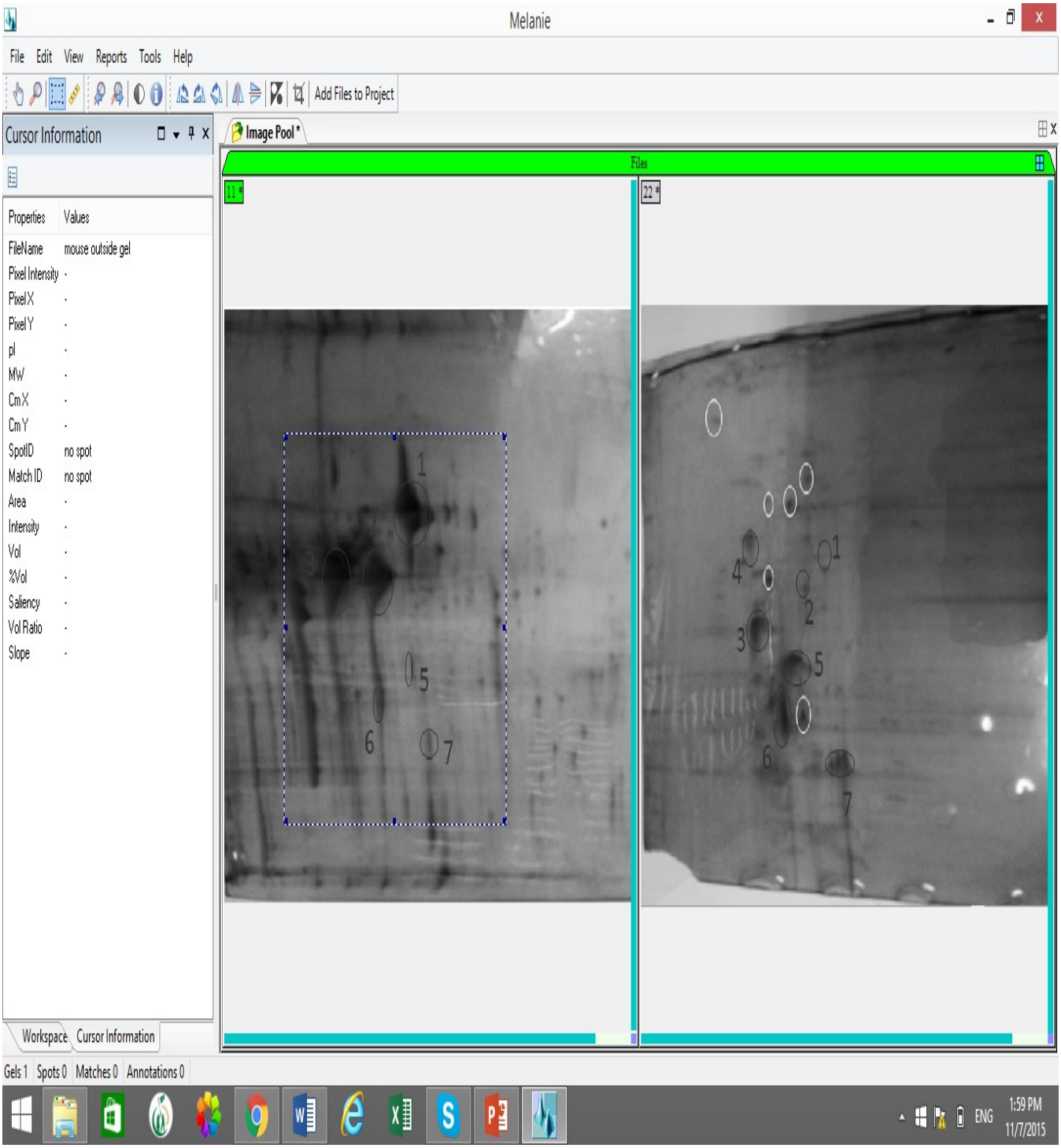

Figure 14. Evaluation of gels in Melanie.

The spots were detected by Melanie and it showed the similar spots with the help of vectors as in figure 14.

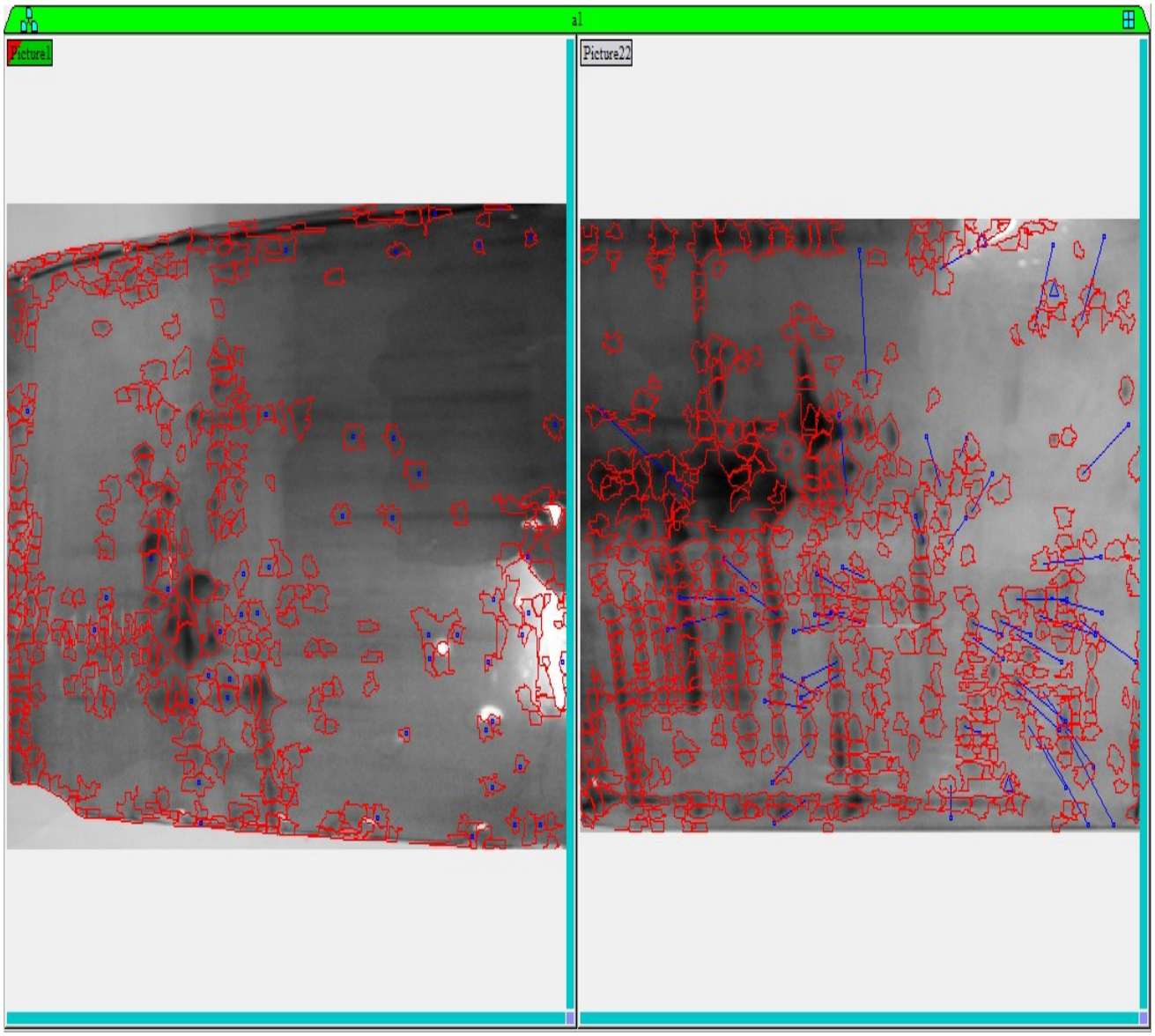

Figure 15 gave similar spots indication.

Figure 15 represents Software reports gave 3D view, match statistic table, gel table, spot table and annotation table. Match statistic table showed 47 similar spots with their central tendencies.

Table 9. Details of similar spots

Match ID"	"Central Tendency"	"Dispersion"	"Coef. Variation"	"Match Count"
0	0.692589	0.58528	0.845062	2
1	0.0504923	0.00136122	0.026959	2
2	0.263674	0.12919	0.489963	2

3	0.138746	0.0639211	0.460706	2
4	0.425789	0.307623	0.722477	2
5	0.183835	0.0738774	0.401867	2
6	0.215946	0.0399635	0.185062	2
7	0.0755632	0.00354721	0.0469437	2
8	0.205616	0.0903628	0.439474	2
9	0.679549	0.454242	0.668446	2
10	0.300158	0.113657	0.378658	2
11	0.216855	0.132588	0.611413	2
12	0.323457	0.116697	0.360782	2
13	0.302537	0.0406904	0.134497	2
14	0.0360249	0.0120012	0.333135	2
15	0.0421607	0.00670526	0.159041	2
16	0.0588093	0.0202977	0.345145	2
17	0.215281	0.106949	0.496789	2
18	0.0988861	0.0437828	0.44276	2
19	0.0916619	0.0244201	0.266415	2
20	2.373	2.19767	0.926115	2
21	0.118213	0.0542092	0.458571	2
22	0.333488	0.263176	0.789161	2
23	0.154242	0.0447232	0.289955	2
24	0.094065	0.00947842	0.100765	2
25	0.435852	0.15653	0.359136	2
26	0.281268	0.0751666	0.267241	2
27	0.0832708	0.00481522	0.057826	2
28	0.503774	0.0224401	0.0445439	2
29	0.0547114	0.0198831	0.363419	2
30	0.079242	0.00410134	0.0517571	2

31	0.17476	1.99646e-4	0.0011424	2
32	0.283716	0.249911	0.880849	2
33	0.139446	0.0370485	0.265683	2
34	0.297798	0.21951	0.737112	2
35	0.148709	0.00771137	0.0518555	2
36	0.121661	0.00547168	0.0449747	2
37	0.181131	0.018773	0.103643	2
38	0.0758217	8.44855e-4	0.0111426	2
39	0.129553	0.053715	0.414619	2
40	0.055805	0.0082855	0.148472	2
41	0.348068	0.242899	0.697849	2
42	0.271828	0.0757179	0.278551	2
43	0.0323408	8.91348e-4	0.0275611	2
44	0.0678936	0.0204662	0.301445	2
45	0.149019	0.0649567	0.435895	2
46	0.166154	0.0739237	0.444911	2

The comparison was done by using different tools. 3 dimensional structure was assessed. There was a significant difference between the spots of cancereous and normal proteins shown in table 9.

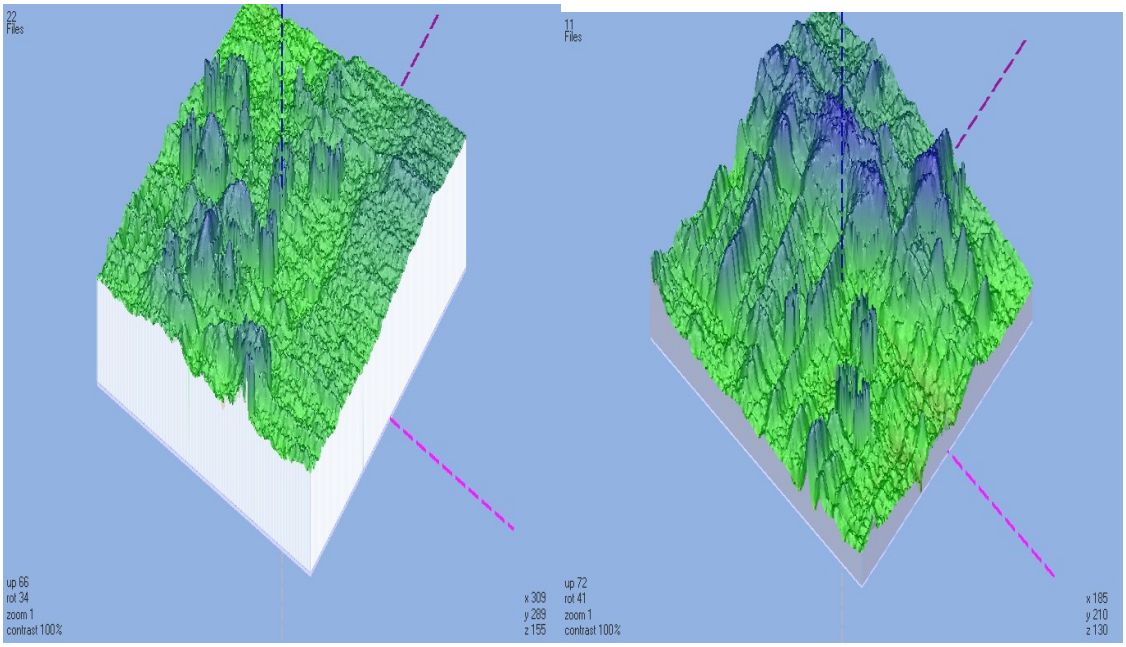

Figure 16. 3D view of area of interest.

7 spots were selected which were highly compareable table 10. The intensities of same spots were compared as indicated in figure 16. Proteins of same molecular weight and distance were matched.

Table 10. Different expression of similar spots.

Spot no	x distance	MW kD	normal intensity	cancer intensity
1	5.6	42	142	208
2	4.9	39	166	207
3	3.6	31	171	212
4	3.3	45	148	174
5	4.7	27	193	160
6	4.3	22	192	126
7	6.1	16	177	133

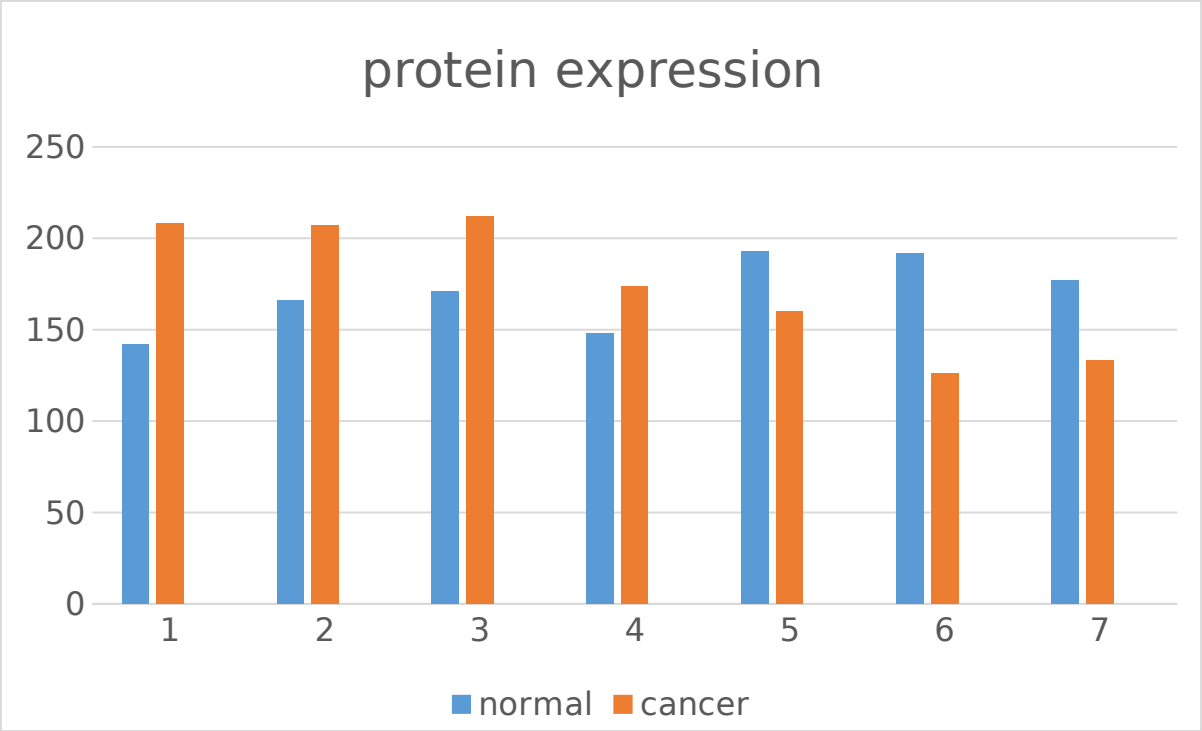

Figure 17. Protein expression in normal and cancer sample.

Figure 17 shows Histogram obtain between 2 gels showed there was upregulation in first four proteins on the other hand last three were down regulated. It can be assessed there is significant difference between the expression of normal and cancerous proteins. In tumor biopsy samples the protein biomarker could be SCCA (45kDa), Heat shock protein 27 (27kDa) and 14-3-3 protein (28-33kDa).

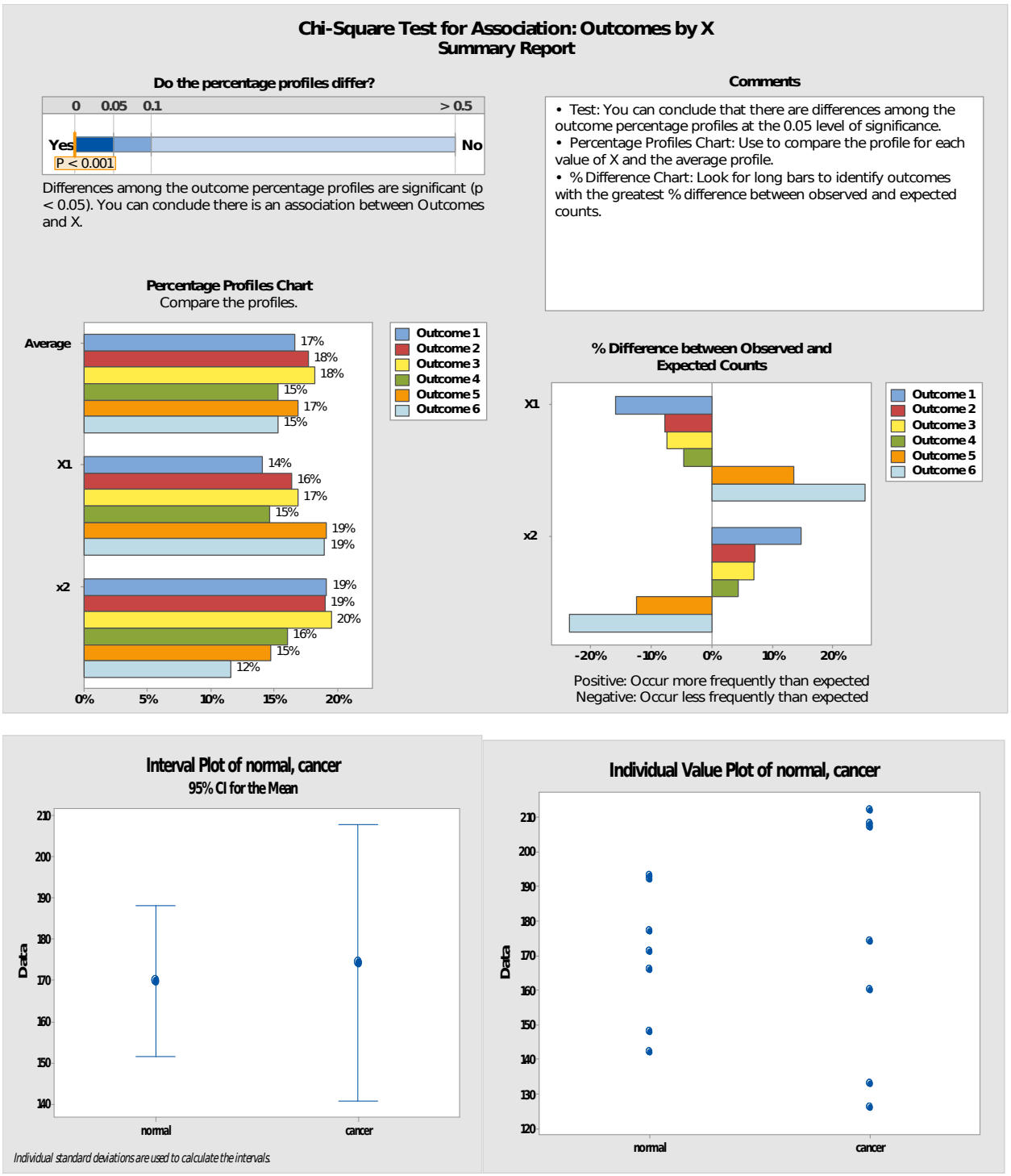

Figure 18. Statistical analysis. Chi-square test prove that protein expression differs significantly in both samples.

When statistic was applied then it can be concluded that the cancerous sample and normal sample proteins are significantly different form one another denoted in figure 18.

Conclusion

HPV is the major cause of cervical cancer among women. The studies shows that its prevalence is high in women of old ages. Moreover, the genotype 16 and genotype 18 are the most prevalent types. When proteomic significance was evaluated for the cancer studies it was concluded that in plasma the protein that significantly expressed different were cytokeratin 19, MMP-9 and complement protein C3. In addition, the SCCA, HSP27 and 14-3-3 protein expression is found to be different in tissue samples. But there is further need of confirmation by using advance proteomic techniques mass spectrometry, immunostaining and western blotting. When the comparison was made between protein expression of cancerous and normal samples. It can be clinched that there is worth able difference between protein expression of cancer cells and normal cells. So proteomics can open insights for cancer diagnosis. Further studies about the role and mechanism of protein can lead to understanding of disease development, diagnosis and treatment.